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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that the annexed is a true copy of the Provisional Specification as filed on 17 June 1998 with an application for Letters Patent number 330710 made by Schlothauer, Ralf-Christian, Schollum, Linda May and Singh, Anne Maria.

Dated 05 July 1999.

Neville Harris
Commissioner of Patents

NEW ZEALAND

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PROVISIONAL SPECIFICATION

DAIRY PROCESS AND PRODUCT

We, RALF-CHRISTIAN SCHLOTHAUER, a German citizen of 14 Colombo Street, Palmerston North, New Zealand; LINDA MAY SCHOLLUM, a New Zealand citizen of 365 Scotts Road, RD 4, Palmerston North, New Zealand; and ANNE MARIA SINGH, an Irish citizen of 8 Sharon Place, Palmerston North, New Zealand, do hereby declare this invention to be described in the following statement:

TECHNICAL FIELD

This invention relates to a process for producing hydrolysed whey protein products which are free of bitter flavours and which contain bioactive peptides. The products of the process have high digestibility and good organoleptic properties. The products may have either a bland or slightly sweet taste and are free of soapy or brothy flavours. The hydrolysed whey protein products may optionally contain oligosaccharides and are useful sources of bioactive peptides for incorporation into functional foods.

BACKGROUND

A number of food ingredients and foodstuffs have been produced from the hydrolysis of a protein source such as the milk proteins, casein and whey proteins.

Hydrolysed protein foodstuffs may have advantages over non-hydrolysed protein foodstuffs in a number of areas of health care. For example, it is known that enzymatically hydrolysed proteins are less allergenic. They are also more rapidly digested and absorbed than whole proteins. Foodstuffs containing hydrolysed proteins are also useful in the alimentation of hospital patients with digestive diseases for example.

Hydrolysis of whey proteins and caseins is known to release bioactive peptides that can exhibit a number of physiological effects (Maubois et al, 1991; EP 475506). A number of publications describe such bioactive peptides, for example, ACE inhibiting peptides which have antihypertensive properties have been released through an enzymatic treatment of bovine β-lactoglobulin and whey protein concentrates (Mullally et al, 1997). ACE inhibitory peptides are also found in sour milk and in hydrolysates of α_s and β casein (JP 4282400; Nakamura et al 1994, Yamamoto et al 1994).

EP 4745506 discloses the hydrolysis of the milk protein lactoferrin in whey to release lactoferricin which acts as an antimicrobial agent useful for treating diarrhoea, athletes foot, eye infections, mastitis etc in humans and animals.

However, the hydrolysis of most food proteins, especially the hydrolysates of whey and casein containing products, is known to generate bitterness. As a consequence the resulting products taste bitter. This causes palatability problems particularly when attempting to formulate orally ingestible products incorporating milk protein hydrolysates as a source of bioactive peptides.

In the field of protein hydrolysis one or both of two approaches are commonly used for controlling or removing bitterness in protein hydrolysates to increase palatability of the products.

The extensive hydrolysis of the protein substrate is known to reduce bitterness in milk protein hydrolysates (EP 065663; EP 117047; US 3970520). Less bitter products are produced relatively easily and cheaply in this way. However, extensive hydrolysis reduces the chain lengths of all peptides, including the bioactive peptides of interest . Extensive hydrolysis of the protein substrate destroys the functional and biological activity of the peptide of interest. In addition soapy and brothy off-flavours often develop, with the consequence that the palatability of the final product remains poor compared to the original bland tasting protein substrate. A final disadvantage is that for some hydrolysates the bitterness is only partially removed (Roy 1992 and 1997).

A second common method for the control of bitterness in protein hydrolysates is to use debittering enzymes, in particular those sourced from *Aspergillus oryzae*.

"Bitterness" generation in protein hydrolysis is thought to be due to the presence of large hydrophobic 'bitter' peptides. Debittering enzymes selectively hydrolyse bitter peptides present in the protein hydrolysates. A worker skilled in the art can - by the judicious selection of debittering enzymes and the conditions of treatment - effectively debitter milk protein hydrolysates leaving intact the particular bioactive peptides of interest. However, use of debittering enzymes makes the process more expensive, and preservation of some of the bioactive peptide is not easily or sucessfully achieved. A further disadvantage is that debittering enzymes treatments have a tendency to release free amino acids into the final product and, as a consequence, the hydrolysates develop unpleasant brothy or soapy flavours (Roy 1992 and 1997).

The various methods of debittering the protein hydrolysates result in additional process steps and add costs to the manufacture of the final product. In addition the final product also becomes overbalanced in its supply of free amino acids.

It would be most adventageous if a process for hydrolysing protein could be developed which releases bioactive peptides of interest and which prevents the formation of bitter peptides and free amino acids, thereby allowing the original bland taste of the milk proteins substrates to be retained.

The bitter flavour developing during enzymatic hydrolysis of milk proteins - up to the point where extensive hydrolysis commences - is generally known to follow the pattern shown in Figure 1a. It is known that the concurrent or subsequent application of a debittering enzyme to the hydrolysate reduces the level of bitter flavours and follows the pattern shown in Figure 1b.

The acceptability of milk protein hydrolysates in relation to flavour forms the pattern shown in Figure 2. Initially, acceptability is high until it drops due to the emergence of bitter flavours (Figure 2a). If debittering enzymes are utilised in the process (Figure 2b) the acceptability recovers, due to the removal of this bitterness, until it drops again due to the generation of brothy and soapy flavours.

Most commercial, extensive milk protein hydrolysates tend to aim for an acceptable range between bitter and brothy flavours, shown as a peak in acceptability in Figure 2b.

Some bioactive peptides - in particular the antihypertensive peptides - are relatively stable during protein hydrolysis and are released very early during the hydrolysis of the milk protein substrate as shown in Figure 3.

The bitter flavours of milk protein hydrolysates can be improved by adding sugars or by hydrolysing natural sugars, such as lactose, already present in the milk protein substrate (Bernal and Jelen, 1989). For example sour wheys and cheese wheys are made more palatable when they have been sweetened by β-galactosidase and lactase hydrolysis of lactose (FR 2309154; US 4358464; JP 8056568).

In order to achieve a high flavour acceptability for a hydrolysed protein product which contains bioactive peptides, precise control of hydrolysis is required to prevent bitterness occurring.

The usual method of termination of hydrolysis is by deactivation of the enzymes, usually by thermal deactivation at high temperatures, typically > 90-100°C . However, this method cannot be used to stop the hydrolysis of whey proteins as any intact unhydrolysed whey proteins remaining in the mixture would denature and precipitate making the final product less soluble and less acceptable for the use as a food ingredient.

It would be advantageous if a process of hydrolysing whey protein could be controlled so that it directly produced a hydrolysate comprising bioactive peptides for incorporation

into functional foods which did not taste bitter and where the enzyme inactivation steps did not compromise the integrity of the intact proteins in the final product.

It is an object of the invention to go some way towards achieving these desiderata or at least to offer the public a useful choice.

It is a further object of one aspect of the present invention to provide a novel whey protein hydrolysate having good organoleptic properties and containing bioactive peptide(s).

It is a further object of another aspect of the invention to provide food containing the hydrolysate of the invention.

SUMMARY OF THE INVENTION

Accordingly the invention may be said broadly to consist in a process for producing a whey protein hydrolysate containing bioactive peptides and having good organoleptic properties comprising the steps:

(i) optional ultrafilter concentration of a whey protein containing substrate using a membrane with a molecular weight cut-off of 5 - 50 kDa;

(ii) treatment of the substrate (if step (i) is not followed) or the retentate with one or more enzymes which hydrolyse the whey proteins;

(iii) termination of hydrolysis without denaturation of intact proteins;

(iv) optional purification of the hydrolysed substrate by subjecting the hydrolysate to either reverse osmosis to remove salt and water or ultrafiltration using a membrane of 5 -100 kDa cut off and recovering the purified hydrolysate,

wherein the one or more enzyme(s) is selected to produce a whey protein hydrolysate containing bioactive peptides and the hydrolysis is terminated before the production of bitter flavours.

The substrate may be selected from the group comprising sweet whey, acid whey, cheese whey, ultrafiltered whey retentate, whey protein isolates, whey protein concentrates, reconstituted whey powder, lactalbumin, reconstituted whey protein concentrates, whey protein fractions, or any other whey protein containing product.

It will be appreciated that some whey products may be contaminated with small amounts of casein protein, depending upon the source and storage conditions, so that the starting material may, in addition to whey protein, contain casein, and the final product may also contain bioactive peptides which have originated from this casein.

Preferably the substrate is sweet whey.

The ultra filter concentration of the whey protein substrate of step (i) is carried out so as to produce an enriched whey protein retentate. Where WPC is used as a starting substrate, this step may not be necessary.

Preferably, the ultra filter concentration of step (i) is carried out so as to produce a partial retentate in which lactose has not been fully removed. In this case, the retentate may be optionally treated with an enzyme(s) to hydrolyse the lactose present into glucose, lactose and/or galacto-oligosaccharides.

The one or more enzymes used to hydrolyse the whey protein in step (ii) are selected as one or more from the group comprising:

food grade enzymes with GRAS and FDA approved status including Fungal proteinases such as those isolated from *Aspergillus oryzae* and *Aspergillus niger* eg Corolase enzymes (Roehm); Protease A and Protease M (Amano Enzymes); Bacterial proteinases such as those isolated from *Lactobaccillus heleveticus, Bacillus licheniformis, Bacillus subtilis* eg Neutrase (Novo Nordisk) and *Lactobacillus lactis*; Animal proteinases eg pancreas proteinases such as Pancreatin, Trypsin and Chymotrypsin; and Plant proteases such as Papain eg Collupullin (Gist Brocades), and any other enzyme having activity on whey and milk proteins as substrates.

Alternatively, a starter culture which produces one or more enzymes which hydrolyse the whey protein substrate may be used. For example, a *L. helveticus* starter culture which is commercially available (Starter Production Unit, NZDRI, Palmerston North, NZ) may be used to hydrolyse a whey protein substrate.

The enzyme hydrolysis step (ii) may be carried out under conditions which are suitable for the particular enzyme or culture used as would be understood by a person skilled in the art.

The whey protein substrates are hydrolysed at a concentration in the range from 5-50% solids and the enzyme or enzyme mixtures are added to give an enzyme to substrate ratio between 0.01% and 3% w/w total solids, preferably between 0.01% and 1.0% w/w total solids.

Protein substrates treated with acid proteases may be hydrolysed at pH between 2.5 and 6.0, preferably between pH 3.0 and 5.0.

Protein substrates treated with neutral proteases may be hydrolysed at pH between 3.5 and 9.0, preferably between pH 6.0 and 8.0.

Protein substartes treated with alkaline proteases may be hydrolysed at pH range between 5 and 10.0, preferably between pH 6.0 and 8.0.

The protein hydrolysis may be carried out at a temperature range of from 20-65°C, preferably from 50-60°C.

The hydrolysis of lactose may be carried out at a prior stage to the whey protein hydrolysis or concurrently thereto. The enzymes used for lactose hydrolysis may comprise lactase and/or β-galactosidase and may be selected from yeast or fungal sources eg *Klyvermyces lactis*, *Saccharomyces lactis*, *Saccharomyces fragillis* or bacterial sources, eg *Aspergillus niger*, *Aspergillus oryzae* such as Maxilact (Gist Brocades) and Novolact (Novo Nordisk). The lactose hydrolysis is carried out under conditions which would be known to persons skilled in the art.

Hydrolysis of the whey protein containing substrate continues for a sufficient time to release bioactive peptides whilst maintaining the organoleptic properties of the hydrolysate, ie, before the production of "bitterness". Once this level of hydrolysis (which is shown in Figure 3 as the "opportunity window") is reached, hydrolysis is terminated.

Termination of the hydrolysis may be achieved by simply deactivating the one or more whey protein enzymes (and/or the lactose hydrolysing enzymes added previously) by firstly changing the pH of the reaction mixture to a pH in which the enzyme(s) is either inactive or less active, and/or heating the reaction mixture to a comparatively mild

temperature using a heat exchanger to denature the enzyme but not the intact whey proteins in the substrate. A suitable temperature range which would denature the enzymes is in the order of 55-70°C, preferable 65°C.

According to one option, depending on the enzyme(s) used, the enzyme or enzyme mixture may also be deactivated by the evaporation and drying procedures.

According to another option the enzyme or enzyme mixture may also be deactivated with or without a prior pH change.

Alternatively, the enzyme(s) may be deactivated with a very short UHT treatment, such as 2 to 10 sec at 90-100°C whereby the enzyme(s) are deactivated before the whey proteins are denatured.

Alternatively, the one or more enzymes used to selectively hydrolyse the whey protein may be immobilised on an inert support such as Roehm Eupergit, Carrageenan particles, chitosan particles or any other suitable material and then used in a stirred tank or fixed bed reactor or on a membrane or on a hollow fiber reactor.

Alternatively, the enzyme(s) to be used for the hydrolysis could be cross linked to suitable inert support prior to the hydrolysis reaction and subsequently separated out of the hydrolysis reaction with the use of a microfiltration membrane.

Alternatively, the enzyme can be separated away from the hydrolysis mixture with the use of an ultrafiltration membrane with a nominal molecular weight cutoff in the range 10 - 500 kDa once hydrolysis is complete.

The hydrolysed retentate of step (iii) may be dried to produce a useful protein enriched product for use as a food additive (see Figure 5). Although this retentate comprises one or more food safe or enzymes approved for food use, such enzymes are in minute quantities, are non-allergenic and are safe for human consumption. In addition the enzymes of this food additive may either be active or have been deactivated as described above. A food additive comprising active enzymes may be useful in the production of yoghurt, for example. Such products contain bioactive peptides such as ACE inhibitory peptides and retain good organoleptic properties and may therefore be used to make more palatable foodstuffs to promote good health.

After hydrolysis and optional deactivation or removal of enzymes, the hydrolysate may optionally be subjected to reverse osmosis in step (iv) under conditions whereby salt and water are removed from the hydrolysate. The purified desalted hydrolysate comprising whey proteins and bioactive peptides is then recovered. If lactose hydrolysis is also chosen then the hydrolysate will also contain glucose, galactose and/or galacto-oligosaccharides.

Optionally the hydrolysed whey proteins containing the bioactive peptide fraction can be separated with a UF membrane of 5-200 kDa cut off, preferably 10-50 kDa cut off. The bioactive peptides, other peptides and, optionally, hydrolysed lactose is recovered in the permeate.

According to another option ion exchange or hydrophobic adsorption or hydrophobic interaction chromatography or combinations of these processes may be used to recover the hydrolysed bioactive fraction from the hydrolysates in an enriched form.

The recovered hydrolysate of steps (iii) and (iv) comprises bioactive peptides of whey proteins which have ACE inhibitor activity, peptides which promote the growth of *Lactococcus* and *Bifido* bacteria and peptides with immune modulating activity (Figure 5).

The recovered hydrolysate has an improved flavour over known hydrolysates. The hydrolysates contain no bitterness. This is especially so when the hydrolysates have been treated with lactase, which converts lactose into the sweet monosaccharides glucose and galactose.

In addition, lactase and β-galactosidase hydrolysis of lactose produces galacto-oligosaccharides which are known to stimulate the growth of beneficial gut flora thereby adding to the bioactive properties of the hydrolysates.

Hydrolysates which have been treated to further hydrolyse lactose are useful as food additives for consumers who are lactose intolerant.

In addition, the residual proteins contained in the hydrolysates of steps (iii) and (iv) are as heat stable as natural WPC in food formulations, which is commercially important for processing the hydrolysate of the present invention.

The process of the invention is summarised in schematic form in Figure 4.

The invention may also be broadly said to consist in an isolated hydrolysed whey protein product which comprises bioactive peptides has good organoleptic properties and has one or more of the following features:

- antihypertensive ACEI activity
- bifidus growth promoting activity
- immune stimulatory activity
- anticariogenic activity
- anti ulcer activity
- non-gluey, non-bitter flavour
- pleasant to slightly sweet taste.

The invention may also be said to broadly consist in foodstuffs incorporating the hydrolysed whey protein product.

This invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, and any or all combinations of any two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which this invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

The invention consists in the foregoing and also envisages constructions of which the following gives examples.

BRIEF DESCRIPTION OF THE DRAWINGS

The present invention will now be described with reference to the accompanying drawings in which:

Figure 1a is a prior art plot showing the development of bitterness with increasing hydrolysis of a protein substrate prior to the point where extensive hydrolysis of the substrate commences;

Figure 1b is a prior art plot showing the change in flavours of Figure 1a when debittering enzymes are added;

Figure 2a is a prior art plot showing the acceptability of a hydrolysed protein substrate with degree of hydrolysis;

Figure 2b is a prior art plot showing the change in acceptability of Figure 2a when debittering enzymes are added;

Figure 3 shows the 'opportunity window' of obtaining a product according to the present invention containing bioactive peptides and having acceptable flavours before the hydrolysis reaction produces bitter peptides;

Figure 4 is a flow diagram showing the various alternative processes which may be used in the process of the present invention; and

Figure 5 is a flow diagram showing the functional foodstuffs available at the different steps of the process of the present invention and properties of the bioactive peptides so produced.

DETAILED DESCRIPTION OF THE INVENTION

As discussed above, the present invention provides a process for producing a hydrolysed whey protein product containing bioactive peptides, whereby the hydrolysis is carried out under a high degree of control to prevent undesirable flavours developing during hydrolysis (eg bitter, soapy and brothy). The hydrolysis is terminated within the "opportunity window", ie before the emergence of bitterness - as shown in Figure 3 - to provide hydrolysates having good organoleptic properties and maximum bioactive peptides.

The combination of mild hydrolysis, i.e. a degree of hydrolysis (DH) of less than 5%, to prevent bitterness, inactivation of enzymes to avoid deactivation of remaining whey proteins, and optional lactose hydrolysis to improve flavour and/or provide biofunctional galacto-oligosaccharides, is novel and results in a superior product to those known in the art.

The present invention is now exemplified by the following three examples:

Example 1

A 10% solution of a sweet whey protein concentrate with 80% protein content (ALACEN™ 392, 2L) was hydrolysed at 50°C with the commercially available enzyme

Neutrase sourced from *Bacillus subtilis* (Novo Nordisk, Denmark). A pH of 7.0 and an enzyme substrate ratio of 0.3% w/w was used for the hydrolysis. The hydrolysate was adjusted to pH 5.0 and heated at 65°C for 30min to inactivate the enzyme. The hydrolysate (DH of 4.5%) was spray dried and tested for angiotensin-converting enzyme (ACE) inhibitor activity and flavour. ACE Inhibitor (ACEI) activity in the dried product was determined using FAPGG as a substrate (Product 305-10 ex Sigma Chemical Corporation, St Lois USA) according of the method of D W Cushman & H S Cheung (1971). ACEI activites are expressed as the amount of material (g/L) needed to reduce the activity of the ACE enzyme by 50%. IC_{50} ACEI activity in the hydrolysate was 0.44g/L and flavour acceptability score, as determined by a taste panel, was very high (8). (Flavour acceptability score 0 = poor; 10 = natural ALACEN™ 392 flavour).

Example 2

A 50% solution of ALACEN™ 421 whey protein concentrate (56% protein content, 10L) was treated with commercial lactase sourced from *Kluveromyces lactis* (Lactozyme 3000L ex Novo Nordisk) at an enzyme to substrate ratio of 0.3% at 50°C for 2 hours. The lactase treated solution was hydrolysed with Neutrase (Novo Nordisk, Denmark) for 1 hour at 50°C at an enzyme substrate ratio of 0.3%. Active enymes were inactivated by UHT treatment (5sec at 95°C) after a five fold dilution of the mixture. The hydrolysate was spray dried. The dry powder (DH 2.8%) contained no traces of active enzyme and had an ACE1 activity of 2.18g/L. The flavour score was exceptionally high (9.8) due to the introduction of a low level of sweetening into the product. ACEI measurements and Flavour acceptability scoring were determined as for Example 1.

Example 3

A 500L hydrolysate, made from ALACEN™ 392 in a similar way to that in example 1, was cooled to 10°C after enzyme inactivation. A sub-sample of the original hydrolysate was dried. The remaining hydrolysate was subjected to ultrafiltration at 10°C with a 10,000 dalton nominal molecular weight cutoff membrane (HFK 131, Koch Membrane Systems, USA). The hydrolysate (at a DH between 3.8% and 4.2%) was concentrated to a VCF 10 and the retentate was dried directly. The permeate was concentrated by evaporation to approx 25% solids and dried. ACEI measurement and Flavour acceptability scoring were determined as for Example 1. The ACEI activity was enriched in the permeate powder (IC_{50} of the permeate powder was 0.15g/L). ACEI activity in the sub-sample of the dried hydrolysate before ultrafiltration was 0.43g/L. The flavour

acceptability scores on the retentate powder and the spray dried powder of the feed were both high (8).

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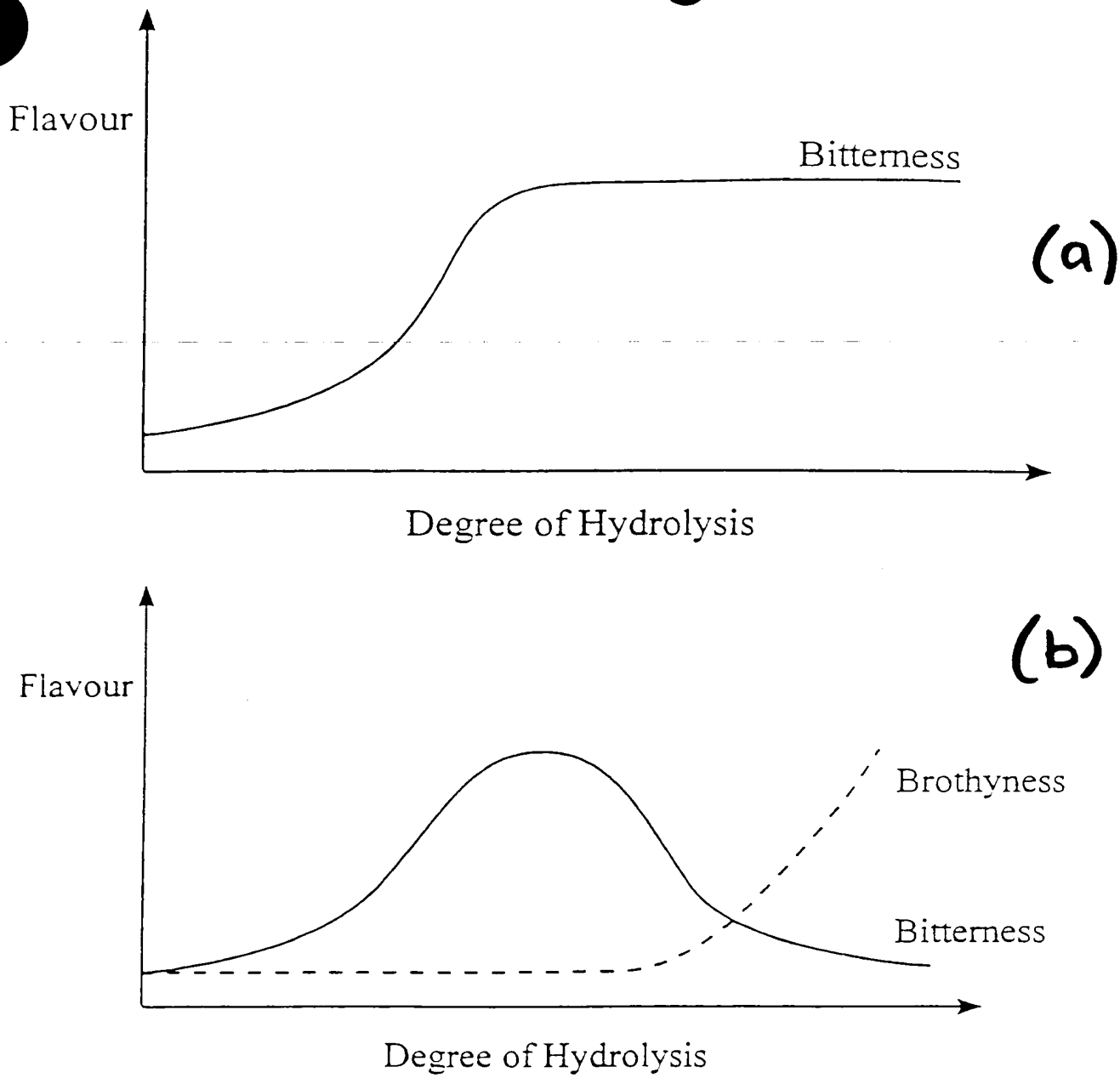
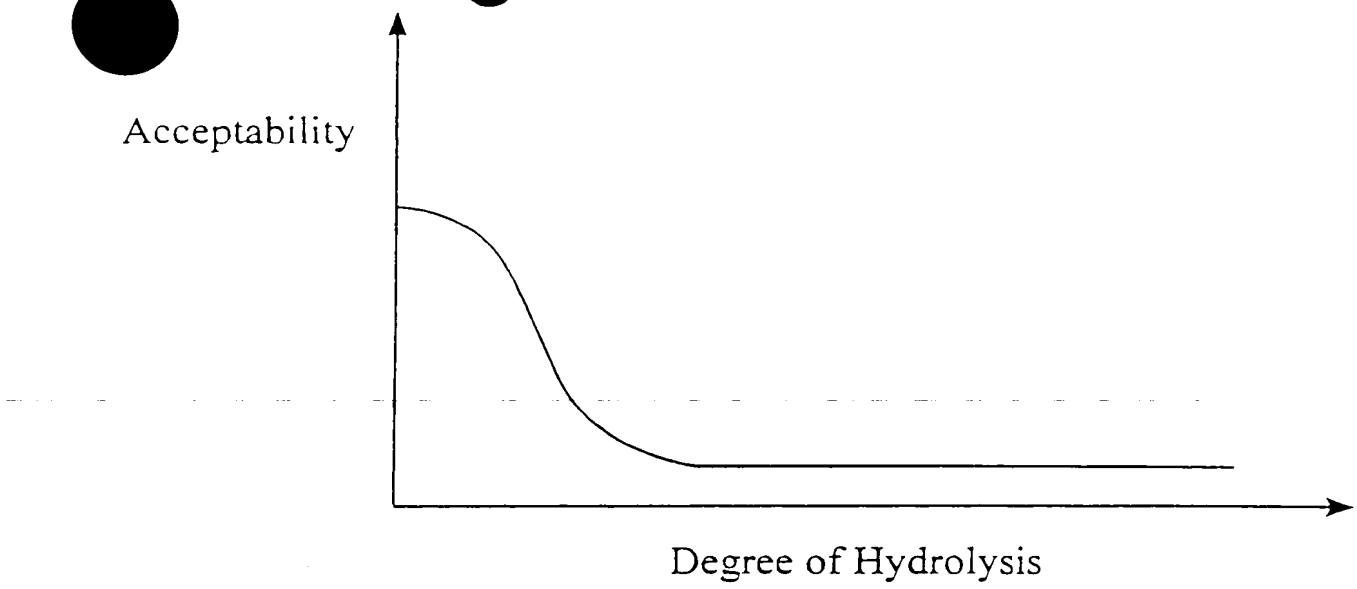
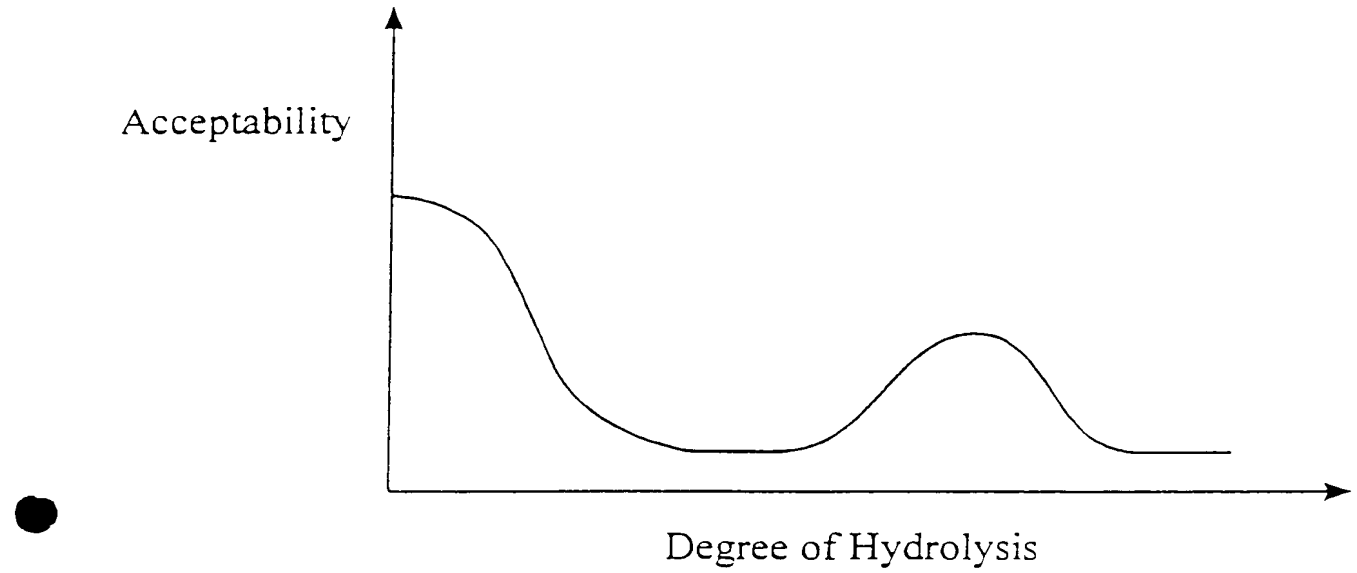

Flavour
Bitterness
(a)
Degree of Hydrolysis
(b)
Flavour
Brothyness
Bitterness
Degree of Hydrolysis

Figure 1


Acceptability
Degree of Hydrolysis

(a)


Acceptability
Degree of Hydrolysis

(b)

Figure 2

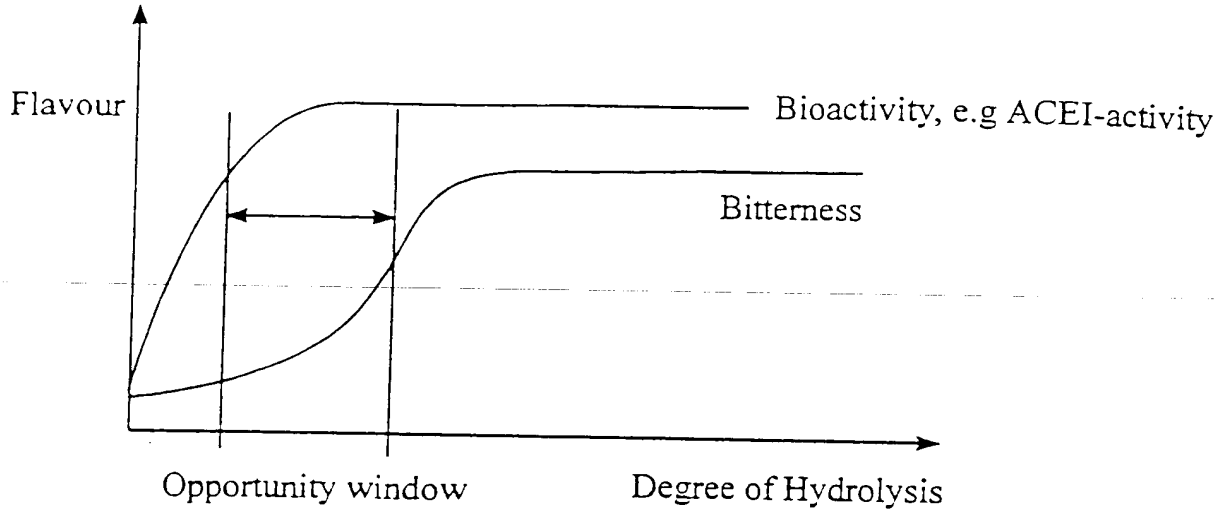

Flavour
Bioactivity, e.g ACEI-activity
Bitterness
Opportunity window
Degree of Hydrolysis

Figure 3

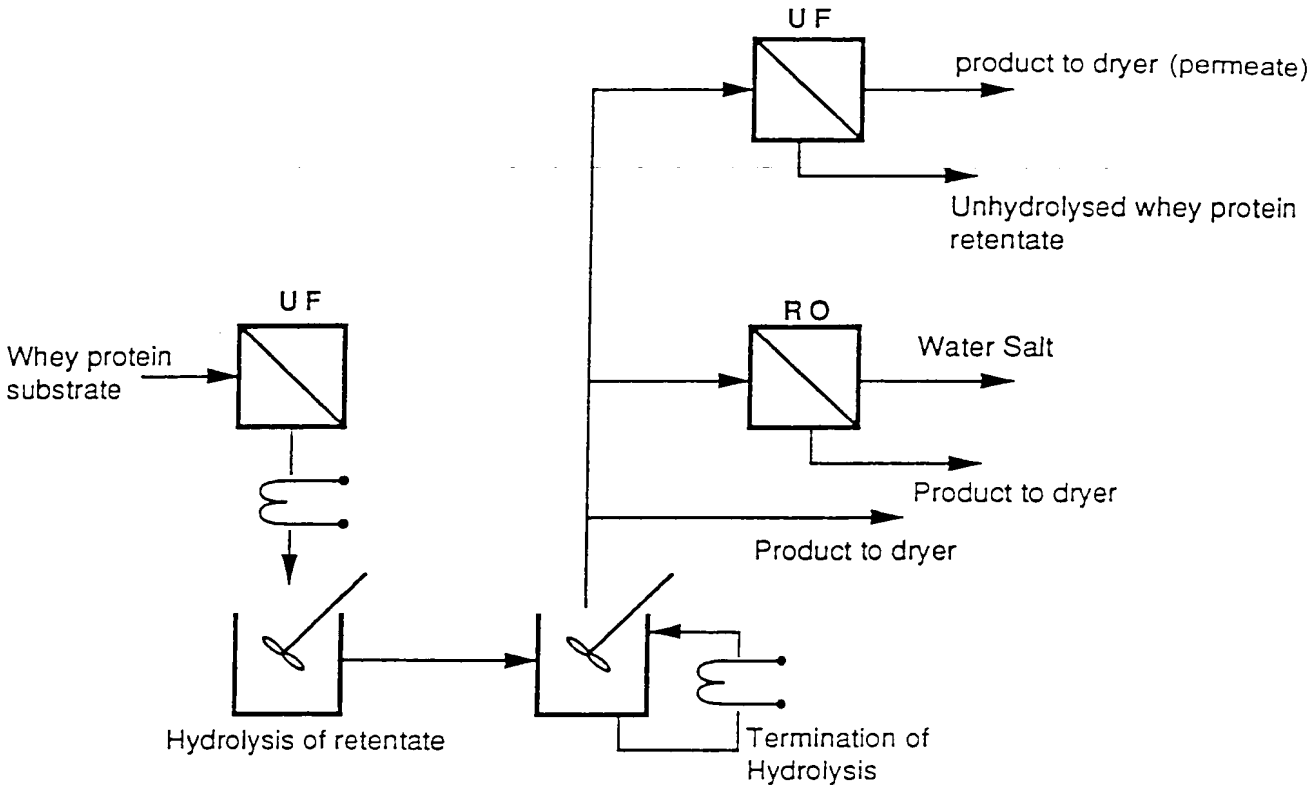

UF
product to dryer (permeate)
Unhydrolysed whey protein retentate
UF
Whey protein substrate
RO
Water Salt
Product to dryer
Product to dryer
Hydrolysis of retentate
Termination of Hydrolysis

Figure 4

Step (I) UF	partial retentate or retentate	enriched whey protein fraction. Possible food additive - no bioactive peptides
Step (ii) Hydrolysis	under precise control to produce maximum level of bioactive peptides before production of bitterness	
Step (iii) Termination of hydrolysis	a) without deactivation of enzymes	enriched protein product containing active GRAS or FDA approved food enzymes. Suitable as a food additive eg for yoghurt, contains a range of bioactive peptides.
	b) with deactivation of enzymes	enriched protein product containing deactivated enzymes. Useful additive for functional foods, containing a range of bioactive peptides.
Step (iv) Purification	a) RO	selectively hydrolysed product containing hydrolysed whey proteins and a range of bioactive peptides. Useful as an additive to functional foodstuffs.
	b) UF	I) permeate comprising hydrolysed whey protein bioactive peptides useful as an additive to functional foodstuffs ii) retentate comprising non-hydrolysed whey proteins - No bioactive peptides. Possible food additive.

* Additionally, hydrolysate products may have been treated to hydrolyse lactose, thereby increasing the organoleptic properties, increasing the bioactive properties (if galacto-oligosaccharides are present) and are useful for lactose intolerant consumers.

Properties of Bioactive Peptides

- ACE I inhibitory activity
- Bifidus growth promoting activity
- Immune stimulatory activity
- Anticariogenic agent
- Anti-ulcer agent
- Protease peptone
- Pleasant non-gluey, non-bitter flavour

Figure 5